ENVIRONMENTAL ENRICHMENT INFLUENCES SOCIAL PREFERENCE TASK BEHAVIOR AND NEURAL ACTIVITY IN ADOLESCENT LONG-EVANS RATS

by

Makayla Renae Wood

Honors Thesis

Appalachian State University

Submitted to the Department of Psychology
and the Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May, 2020

Approved by:

Mark C Zrull

Mark C. Zrull, Ph.D., Thesis Director

Nicholas N. Shaw, Ph.D., Second Reader

Andrew Smith

Andrew R. Smith, Ph.D., Departmental Honors Director

Jefford B. Vahlbusch, Ph.D., Dean, The Honors College

Abstract

Adolescence is a time of physiological growth and development in human beings featuring enhanced social interaction, risk-seeking behavior, and curiosity. Adolescent rats exhibit similar, species-specific characteristics. The aim of the current study was to observe changes in behavior and neural activity in adolescent rats through environmental manipulation. Environmental Enrichment (EE) is the stimulation of social and physical aspects of a laboratory animal's environment and can lead to various neurological and behavioral advancements. Subjects in this study were 24 Long-Evans rats that were divided into two conditions: experimental and control. The experimental group experienced EE sessions and the control group did not. After 20 EE sessions, rats from both groups participated in a social preference task (SPT). The SPT is a two-trial procedure commonly performed to analyze how EE affects the inclination of a rat to act toward a familiar or novel stimulus rat. Once behavior was observed, rats were euthanized and brain tissue was processed to identify neurons activated by the SPT using a neural activity marker, c-FOS. The basolateral amygdala (BLA) and the hippocampus' *Cornu Ammonis* 2 (CA2) were regions of interest. Combined, these areas are crucial for fear processing and social learning memory. Results showed that neither the EE nor the control rats displayed a significant preference for either stimulus rat during the SPT. It was, however, established that compared to other conditions, male enriched rats spent a significantly greater amount of total time ($p < .001$) and time per contact ($p < .001$) with either stimulus rat. Additionally, the BLA of enriched rats had greater mean neural activity than the CA2 of enriched rats ($p = .008$) and the BLA of non-enriched rats ($p = .005$). There was no significant evoked activity in the CA2 of either group. Overall, results suggest EE may decrease an adolescent rat's probability of engaging in risky behavior in a social setting through experience allowing for informal learning and memory that

may promote neurogenesis and synaptic plasticity. It is thought that analogous strategies could be employed to impact adolescent behavior and neurological structure in people.

Keywords: adolescence, enrichment, social preference, neural activity, amygdala, hippocampus

Environmental Enrichment Influences Social Preference Task Behavior and Neural Activity in Adolescent Long-Evans Rats

During adolescence, human beings experience a number of essential physiological and psychosocial changes. This period of time is typically characterized by heightened interactions with peers, boundary-testing behavior, and increased curiosity. It is suggested these changes are due to developments in the social brain (Kilford et al., 2016). Because of these physiological and psychosocial changes, human adolescents are likely to possess complex personality traits and thought processes, as well as greater emotion and social memory formation. Heightened peer interaction, boundary-testing behavior, and increased curiosity ultimately lead to risky decision making and potentially threatening social engagements. Adolescence in laboratory animals, specifically rats, has been found to provide analogous social brain developments. Burke et al. (2017) suggests that adolescent rats are highly social and curious creatures. During this stage, rats are more inclined to explore and interact with their surroundings and litter mates. Risky behavior in these animals can be manipulated and observed in the laboratory by introducing novel environments, various stimuli, and opportunity to interact with conspecifics.

Environmental enrichment (EE) provides an opportunity to foster physical and social stimulation, in that laboratory animals are exposed to complex floor plans, unique objects, and cage mates (Simpson & Kelly, 2011). This type of environment plays a large role in the laboratory animal's inclination to engage with social and physical novelty. A procedure known as the social preference task (SPT) can be used to measure social behavior in rats that may be analogous to risky behaviors in humans. Specifically, the SPT measures a subject's propensity to spend time with a familiar or unfamiliar counterpart. In a study conducted by Moy et al. (2004), it was discovered that in general, non-enriched adolescent rodents demonstrated a strong

preference for the novel conspecific, rather than the familiar conspecific. Significant preferences for novelty in social interaction were determined by length of time spent with the unfamiliar rodent and its degree of overall activity (Moy et al., 2004). The inclination to spend more time with a novel rat suggests the tested animals did engage in risky behavior as adolescents. The current study seeks to investigate whether this preference for social novelty changes with an EE history.

Because EE exposes laboratory animals to a variety of unfamiliar conditions, the technique has been found to cultivate greater levels of neurological and behavioral stimulation compared to standard housing conditions (Simpson & Kelly, 2011). It is thought that EE influences neural activity in various brain regions due to the unique function any brain region has during experiences of novelty. The hippocampus and amygdala should be thought of as particularly important during novel experiences due to the complex role that emotion and memories have on decision making, especially during adolescence. It is suggested that risky behavior in rats is associated with increased neural activity in both of these areas (Simpson & Kelly, 2011). The current study is an investigation of the significant impact that EE has on these brain regions, SPT, and the role hippocampus and amygdala may play for an animal in the SPT.

Human Adolescence and Social Interaction

Adolescence in human beings – commonly identified as the period between childhood and adulthood, i.e. ages 10 to 19 – is a particularly important course of transition as it marks an essential time for the development of many social attributes that are critical for traditional human life. During adolescent years, humans commonly exhibit behaviors and learning styles that are based primarily on emotion. Guyer et al. (2016) review a number of studies and suggest that adolescents have a greater range of emotions, greater frequency of emotions, and shorter

individual emotional duration than children or adults. Because of this, adolescents tend to be more responsive to social events that in turn generate behavioral responses, facilitate learning, and help shape adaptive response patterns. These responses to social events can be either positive or negative and rely primarily on the context of the event itself. King et al. (2018) conducted a study on how the nature and quality of peer interactions influence self-regulation in adolescents. They found that peer exposure during social events increases the occurrence of risk taking or risky decision making among adolescents, especially if the peer interaction was positive. Because of this, adolescents begin to associate positive experiences with risk taking which reinforces such behavior (King et al., 2018).

Rat Adolescence and Social Interaction

Adolescence in rats can be identified as extending from postnatal day (PND) 21 to 59. More specific classifications include: juvenile or early adolescence (PND 21 to 34), middle adolescence (PND 34 to 46), and late adolescence (PND 46 to 59) (Laviola et al., 2003). During this time, rats exhibit neurological changes that encourage behavior patterns such as greater frequency and duration of social interactions, peak levels of play, and increased exploration and novelty-seeking (Spear, 2000). Rats seem to more successfully negotiate the developmental transitions associated with adolescence when activities like exploration and novelty-seeking are performed with litter mates rather than when said activities are performed alone (Spear, 2000). Additionally, researchers Meaney and Stewart (1981) suggest that social interaction during adolescence allows rats to develop appropriate patterns of behavioral response to stimuli, or cues, from conspecifics. These behavioral responses may include characteristics of play such as pouncing, boxing, on-the-back posture, on-top posture, or neck grooming. In addition, Marci et al. (2002) found that compared to juvenile and adult aged rats, adolescents (i.e., PND 30 to 59)

spent a greater proportion of time investigating the open arms of the plus-maze where animals have the opportunity to venture out onto the open, wall-less platforms of an elevated apparatus. Researchers interpreted this behavior as increased risk-seeking and exploratory drive, or decreased environmental-related anxiety. These findings are in line with what has been discovered in adolescent human beings.

The rat remains a favorable species for modeling events or time periods also observed in humans due to similarity in neural development, cytoarchitecture, and neural chemistries (Andrzejewski et al., 2011; Parker et al., 2014; Smith et al., 2019). Much is known about the rat's learning capabilities which allows for predictions to be made about future behaviors. Additionally, rats are easily bred and their ability to be tested in a wide range of environments provides for optimal research manipulation (Andrzejewski et al., 2011; Parker et al., 2014).

Environmental Enrichment

EE can be defined as the stimulation of social and physical aspects of a laboratory animal's environment and has been suggested to lead to various neurological and behavioral advancements (Simpson & Kelly, 2011). These environmental manipulations are considered significantly more complex and arousing than what the animal might experience under regular housing conditions. Physical enrichment of cages includes the involvement of toys, ramps, ladders, running wheels, structural modifications, etc., whereas social enrichment involves caging multiple litter mates, or sex- and age-matched non-litter mates, together as a source of interaction. A combination of both types of enrichment is preferred and most effective (Simpson & Kelly, 2011).

Simpson and Kelly (2011) suggest that EE enhances neural plasticity of laboratory animals, which ultimately aids in the production of learning and memory. Neural plasticity can

be defined as the proficiency with which the nervous system develops and evolves in response to and accordance with environmental diversity. This is typically accomplished through synaptic change, synaptogenesis, and/or neurogenesis and neural proliferation. Other characteristics associated with EE and plasticity include an increase in overall brain volume, synaptic growth, and cortical thickness (Simpson & Kelly, 2011). Mora et al. (2007) found that environmentally enriched rats exhibited greater levels of neurogenesis in multiple areas of the hippocampus when compared to rats living in control housing conditions. This may be due to advancements in learning and memory taking place during times of enrichment. More specifically, these areas displayed increased concentration of granule cells and neurotransmitters glutamate and GABA (Mora et al., 2007). Due to the sweeping effect these neurotransmitters can have throughout the nervous system, it can be suggested that EE-induced changes are global with the inclusion of experience-dependent plasticity of pre-existing circuits.

Simpson and Kelly (2011) also found that EE allows laboratory animals to better adapt to future novel environments as they become more accustomed to and expectant of change. They are able to explore their surroundings and interact with their cage mates more freely and at their own will. In a study performed by Brenes et al. (2008), it was shown that compared to controls EE rats exhibited lower levels of initial locomotor activity and higher levels of grooming upon placement in a novel testing environment. This indicates that rats accustomed to arousing environments are quicker to adapt and accept their new surroundings with suppressed initial curiosity, as indicated by their immediate engagement in ordinary grooming activities (Brenes et al., 2008).

EE creates an overall learning environment that has the ability to increase the likelihood of adaptation to novel experiences if repeated exposure is involved, especially during

adolescence. Becoming familiar to these experiences increases complex thought processes and development of emotion-based decision making of rats through the stimulation of the amygdala (Stansfield & Kirstein, 2006). Connections between the hippocampus and the amygdala link emotion to memories and vice versa through reinforced dendritic branching. Prolonged exposure to EE allows for long-term impacts on the brain to happen and be observed (Stansfield & Kirstein, 2006). It may be meaningful to summarize that in order for a rat to fully engage in EE, it would be ideally an adolescent as they are more willing to explore new environments compared to juvenile or adult rats. Subsequent to EE exposure, risky behavior is seen to decrease in adolescent rats, as the animal no longer expresses an arousing response to novel stimuli due to neural development promoted by EE.

Social Preference Task

The SPT is a two-trial procedure commonly performed in the laboratory and can be used to analyze how EE affects the inclination of an animal, in this case a rat, to act more favorably towards one conspecific over another. In the current study, stimulus rats were the conspecifics of interest, which is further discussed in the materials and methods. Risky social behavior in a rat can be identified as favoring an unfamiliar stimulus rat over a familiar stimulus rat, with favoring operationalized as more time spent or more contacts made. Enriched animals are thought to adapt to novel stimuli at a faster rate than unenriched conspecifics (Simpson & Kelly, 2011). Due to this observation, EE should have the ability to influence the SPT such that enriched rats would be more likely to become quickly habituated to a novel stimulus rat. This may result in a lower number of contacts or total amount of interaction time the enriched animal spends with the novel stimulus rat as their initial curiosity is lower than as for a non-enriched rat (Simpson & Kelly, 2011). Such a result would be consistent with the findings by Brenes et al. (2008) that describe

EE rats as exhibiting lower levels of initial curiosity when placed in a novel testing environment than non-enriched rats, as indicated by the enriched rats' immediate engagement in ordinary grooming activities rather than exploratory behavior.

Woods (1962) suggested that perception of novelty is not only determined by an animal's current environment, but also their immediate prior housing arrangements. Because of this, Woods (1962) concludes that stimulus complexity plays a large role in the behavioral response of enriched animals during a preference task such that an enriched animal may perceive a novel stimulus as less than or only equally as arousing as items found during its past encounters. Thus, the enriched rat will not show preference toward the novel stimulus; i.e., it will not engage in behavior that a researcher might consider risky. As mentioned by Simpson and Kelly (2011), synaptic development is heightened during adolescent EE, meaning that a rat's ability to learn and make memories of their previous environments and/or stimuli is strengthened. The enhancement of synaptogenesis or synapse rearrangement plays a large role in how an animal reacts to its surroundings, such that rats with history of enrichment, and related synaptic change, may need greater levels or environmental or social arousal to stimulate their interest in novel stimuli.

Amygdala and Hippocampus

The rat amygdala plays a crucial role in the corticolimbic circuit by responding to stimulus driven input and producing output that modifies activity through various pathways that function to regulate specific emotions and motivation. In order to process such information, the amygdala must first receive signaling cues from the external environment through the sensory thalamus and sensory cortices (Janak & Tye, 2015). These signals are transmitted to the amygdala's input region, lateral amygdala (LA), as afferent visual, auditory, or somatosensory

information. Next, these signals are either conveyed to the amygdala's integration region, basolateral amygdala (BLA), or bypassed to the amygdala's output region, central amygdala (CeA) (Janak & Tye, 2015). Bypassing to CeA allows raw stimuli to activate amygdala output without additional processing in BLA and thus produce a more rapid behavioral or physiological response (Sah et al., 2003). CeA ultimately functions to activate efferent projections as the primary output structure of the amygdala, receiving information from both LA and BLA (Sah et al., 2003).

Although the amygdala is composed of multiple interconnected components, BLA is the focus of the current study. The neurons of BLA have many important neural connections with the brain's cortices, allowing for integration of various sensory inputs to produce an appropriate behavioral response, although not an immediate response (McDonald, 1982). Specifically, BLA has been found to play a vital role in the modulation of memory consolidation in Pavlovian fear training (Vazdarjanova & McGaugh, 1999). This is important when considering an animal's probability to interact with novel or threatening stimuli, which may be labeled as risky behavior. BLA is also interconnected with the hippocampus (HPC), and it can receive both sensory and contextual information and facilitate integration of that information. There is a bridge between BLA and HPC that allows for this contextual integration to occur, which provides the mechanism to add an emotional component to memories and increase the significance and importance of these memories (Sah et al., 2003). Figure 1 demonstrates the amygdala circuitry.

The rat HPC is critical for a majority of the animal's learning and memory processes. Most of the information that is processed by the HPC is from the cortices (Boccara et al., 2015). Cortical information that reaches the HPC is transmitted to the HPC's input region, the dentate gyrus (DG). These signals are then conveyed through multiple pyramidal cell regions called

Cornu Ammonis 1, 2, and 3 (CA3 → CA2 → CA1), with each structure playing a unique role in memory formation. Finally, the signals reach the HPC's main output center, the subiculum (Jarrard, 1993). As a whole, the HPC functions as an initial processing center for contextual information, which has been shown in lesion experiments (Jarrard, 1995). Results demonstrated that the HPC allows animals to utilize contextual stimuli and tailor their response according to contextual clues.

Although the HPC operates as a product of a complex circuit of subunits, the current study focuses solely on CA2, a small but distinct region significant for spatial, contextual, and social memory of animals (Alexander et al., 2015). Because of these functions, CA2 is the most sensitive of all pyramidal cell regions for remapping familiar environments with the addition of novel stimuli (Alexander et al., 2015), which is why it holds the utmost concern for studies involving EE and SPT during which novel stimuli are other animals. Stevenson and Caldwell (2014) proposed that although CA2 is capable of performing many functions, its most important and relevant role is that of forming social memories. Through lesion studies, these researchers found that formation of social memories is partially olfactory-based with CA2 neurons receiving input from the entorhinal cortex, which processes some olfactory information. Stevenson and Caldwell (2014) state this olfactory component is important for determining the 'what' and 'when' of contextual stimuli, which is in turn useful in the consolidation of social memories.

c-FOS

c-FOS is a protein and neural activity marker that can allow for the mapping of functional activity in amygdala and hippocampal sub-regions, exposing areas with heightened neural activity. Active neurons are associated with a dark color due to heavy c-FOS accumulation, whereas inactive neurons are associated with a lighter color, or are not seen at all, due to lack of

c-FOS. This protein marker is derived from the immediate early gene (IEG), *c-fos*, which precedes the signal transduction sequence to affirm the production of the c-FOS protein (Chaudhuri, 1997). This sequence is most commonly initiated by calcium influx into the cell, which either takes place through a NMDA receptor complex after glutamate binding occurs, or through voltage-sensitive calcium channels (Chaudhuri, 1997). From here, various enzymes systems are activated with the presence of calcium. Multiple signaling pathways transpire to then initiate transcription factors, and ultimately transcribe the IEG into c-*fos* mRNA, which is finally translated into the c-FOS protein (Chaudhuri, 1997). This neural protein marker has been found effective in a number of studies for identification of neural activity (Bullitt, 1990; Herrera & Robertson, 1996; Sagar et al., 1988), and was therefore employed in the current investigation.

Current Study

The purpose of the current study is to illuminate the effect of EE on social preference of adolescent rats as well as on neural activity within the BLA and CA2 region of hippocampus evoked by SPT. It was anticipated that repeated exposure to EE during adolescence would significantly influence a rat's behavior and associated neural activity when compared to non-enriched controls.

Based on the social preference literature, specifically Brenes et al. (2008), Moy et al. (2004), and Woods (1962), a lesser amount of engagement with the novel conspecific in a SPT from an enriched than a control animal was expected. Specifically, it was hypothesized that while adolescent rats with a history of enrichment would show no preference to either stimulus rat during the SPT, the non-enriched adolescent control rats would show preference to the novel stimulus rat.

Considering particularly the findings of Simpson and Kelly (2011), it was predicted that enriched rats would display increased neural activity in the BLA and CA2 region of hippocampus during social interaction due to prior experience in a similar setting (i.e., during EE sessions). Precisely, it was hypothesized that adolescent rats with a history of enrichment would have a significantly greater number of neurons in BLA and CA2 activated by performance of the SPT than adolescent control rats.

Materials and Methods

Subjects and Experimental Design

All animals used in the study were cared for in the Arts and Sciences Animal Facility at Appalachian State University. Subjects (N=24) were equal numbers of male and female Long-Evans rats aged 22 to 49 days over the course of the study. They were housed in standard plastic shoebox cages in a humidity and temperature controlled vivarium on a 12 h on/12 h off light-dark schedule. Subjects lived in same-sex groups of three and were provided food and water *ad libitum*. Subjects were randomly assigned to either the experimental or control group, each consisting of 12 animals (6 male, 6 female). The experimental group received 90 min enrichment sessions on 20 days, while the control group remained in standard housing. Control rats were picked up and put down twice on days that experimental group rats were enriched.

Six additional age-matched Long-Evans rats were used as stimulus animals (3 male, 3 female); an important component of the SPT used in the study. The SPT was conducted on PND 49 after the 20 sessions of environmental enrichment or handling for the control rats. After the SPT, rats were sacrificed and brain tissue was processed for further analysis. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University (Protocols #15-02 and #19-10, M. C. Zrull, P. I.).

Environmental Enrichment

Rats in the experimental group were placed into multi-level, gender specific enrichment cages for 90 min sessions on 20 occasions between PND 22 and 48. Cages were made of wood and wire mesh with dimensions of 46 X 48 X 79 cm (w X d X h). Cage platforms were located 14, 25, 43, and 61 cm above the base, which was covered in aspen bedding. Toys were placed on the cage base, multi-level platforms, and hung from the ceiling (see Figure 2). The purpose of introducing toys and other inanimate objects into the enrichment cages was in effort to stimulate exploration and play. Male and female cages mirrored one another in platform structure and toy layout. Toys were kept in separate gender specific boxes when not in use to avoid scent from the opposite sex interfering with the rats' experience. Toys and toy arrangements were rotated through four set-ups across the 20 sessions. In addition to play time with toys, rats were also able to interact with conspecifics during their enrichment sessions. Subjects in the control condition stayed in regular shoebox housing conditions for the duration of their lives, but were picked up and put back down to control for the experimental group's handling when loaded into and unloaded from the enrichment cage.

Social Preference Task

The SPT was a two-trial task, with both trials conducted in a 63 X 42 X 45 cm (w X d X h) wooden box with aspen bedding on the floor (see Figure 3). Each trial lasted 3 min. During Trial 1, an additional experimental or control rat was paired with an unfamiliar stimulus rat (Stimulus Rat A) for 3 min. Following Trial 1, there was a 30 min delay before Trial 2. The second trial consisted of the two rats from Trial 1 as well as a third, novel stimulus rat (i.e., Stimulus Rat B) interacting for 3 min. For each trial, the number of times the only or either stimulus rat was contacted by the experimental or control rat was recorded as well as the duration

(in seconds) of each interaction. Contact included grooming, play, nuzzling, chasing, pouncing, etc. In order to be counted, the contact had to be deliberate and not initiated by the only or either stimulus rat. All SPT trials were videotaped for additional and reliability analyses.

Histology

Following Trial 2 of the SPT, each experimental or control subject was placed into quiet and dark containment for 90 min to allow for expression of the immediate early gene *c-fos* and adequate production of c-FOS protein, a neural activity marker. After this period of time, each animal was injected with a lethal dose of sodium pentobarbital (100 mg/kg b.w., ip). Upon absence of a rat's corneal and tail reflexes, subjects were perfused intracardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 10 mM phosphate buffer (PB). Then, the head was removed using a small guillotine. Each brain was dissected out of the cranium and post-fixed in a 10% sucrose, 4% paraformaldehyde in PB solution at 4 ºC for 5 days. All brains were stored in 10 mM PB with 0.02% sodium azide at 4 ºC until processed.

Brains were divided into left and right hemispheres and subsequently cut into sagittal sections at 50 μm using a Vibratome Series 1000 Sectioning System. Sections of interest were processed using floating section immunohistochemistry (IHC) to observe c-FOS in the nuclei of neurons activated by the SPT. On day one of IHC, sections were in PBS (2 x 5 min) and incubated in 15% goat serum with 0.2% Triton-X for 60 min. Following this, rat anti-c-Fos made in rabbit (Santa Cruz Biotechnology, SC-52) was used to process sections at 4 ºC for 40 h. On day two of IHC, sections were rinsed once more in PBS (6 x 10 min) and incubated in biotinylated goat anti-rabbit secondary antibody for 60 min (Vector). Next, sections were rinsed in PBS (3 x 10 min), exposed to a peroxidase labeled avidin-biotin complex for 60 min (Vector), and rinsed again in PBS (2 x 10 min). Finally, an enzyme substrate (VIP, Vector) was applied to

sections for at least 2 min. Sections were mounted onto gel-coated slides and air-dried. They were then dehydrated in graded ethanols, cleared with toluene, and cover-slipped with Permount (Fisher). In order to visualize cytoarchitecture, alternate sections were processed for Nissl staining with thionin.

Microscopy and Data Analysis

Activated neural nuclei in CA2 and BLA, as indicated by the presence of c-FOS, were identified and compared between the two conditions. Brain sections were examined using a Nikon Eclipse microscope with a Plan 10 objective, 1.3 megapixel PixeLink camera, and a 1024 X 768 pixel image. Using two atlases of the rat brain (Paxinos & Watson, 1998; Pellegrino et al., 1979) as guides, CA2 and BLA could be identified in all tissue samples. Digital photographs were taken, and further analysis was done using the Adobe Photoshop application on a computer. A scaled grid was applied to each photo; counters identified a specified number of 200 X 200 μm square sections within each brain region. The Photoshop counting tool was then used to count the number of darkened neural nuclei within each grid section, from right to left. A digital image of c-FOS containing nuclei was used by counters to determine, as objectively as possible, if any particular nucleus was dark enough to be counted as indicating an active neuron. To prevent counting the same neuron twice, in multiple grid samples, those activated neurons located on the left and bottom lines defining each 200 X 200 μm square were not counted. To ensure accurate quantification, all images were counted by two observers who were blind to the condition. Neuron counts were averaged across counters.

Results

Social Preference Task

Hypothesis 1 stated that while adolescent rats with a history of enrichment would show no preference to either stimulus rat during the SPT, the non-enriched adolescent control rats would show preference to the novel stimulus rat. This hypothesis was partially supported (see Table 1). As predicted, there was no significant difference in the amount of time enriched rats spent interacting with either stimulus rat. However, on average, EE rats did spend a greater amount (+41%) of time interacting with the familiar stimulus rat (M = 12.17, SD = 5.20) than they did with the novel (M = 7.83, SD = 5.61), but this finding lacked statistical significance, $t(22) = 1.961$, $p = .063$. Additionally, non-enriched rats did not show preference to the novel stimulus rat. Results showed no significant difference in the amount of time non-enriched adolescents spent with the familiar stimulus rat (M = 9.42, SD = 3.00) versus the novel stimulus rat (M = 8.67, SD = 4.74), $t(22) = 0.46$, $p = .650$. See Figure 4 for a graphed display of the mean time spent with the familiar and novel stimulus rats for both conditions.

Albeit not the study's primary interest, a significant sex difference was found when considering total interaction time with stimulus rats (see Table 2) and the duration of each individual contact with either stimulus rat (see Table 3). Enriched adolescent males spent significantly more time (+198%) interacting with either stimulus rat during Trial 2 (M = 44.08, SD = 22.19) than enriched females (M = 16.25, SD = 6.90), $t(22) = 4.1486$, $p < .001$. Enriched adolescent males also had significantly longer durations (+176%) of each contact during Trial 2 (M = 4.94, SD = 2.25) than enriched females (M = 2.03, SD = 0.91), $t(22) = 4.1532$, $p < .001$. See Figure 5 and Figure 6, respectively, for graphed display of mean total interaction time and mean time per contact spent with either stimulus rat across conditions and sexes.

Amygdala and Hippocampus

Hypothesis 2 stated that adolescent rats with a history of enrichment would have a significantly greater number of neurons in BLA and CA2 activated by performance of the SPT than adolescent control rats. This hypothesis was partially supported (see Table 4). The number of active neurons in BLA of enriched rats (M = 23.75, SD = 12.41) was significantly greater (+201%) than the number of active neurons in BLA of control rats (M = 7.88, SD = 2.26), $t(14)$ = 3.33, p = .005 (see Figure 7). The number of active neurons in CA2 of enriched rats (M = 9.00, SD = 2.92), however, was very similar (+16%) to the number of active neurons in CA2 of control rats (M = 7.76, SD = 2.22), $t(14)$ = 0.9022, p = .380 (see Figure 8). An additional finding was that enriched rats exhibited a significantly greater amount (+164%) of active neurons in BLA than in CA2, $t(14)$ = 3.0619, p = .008 (see Figure 9). Sex differences were not accounted for in the examination of neural activity evoked by the SPT. Figure 10 shows a graphed display of mean neural densities across both conditions for BLA and CA2.

Discussion

The objective of this study was to determine the impact that EE has on social preference behavior and related neural activity of adolescent Long-Evans rats. Because adolescence is a time of great vulnerability (Spear, 2000), it was thought that substantial and significant changes would be observed in both areas of interest for rats with a history of enrichment. Because the impact that EE has on an adolescent animal could be long-term and allow continued advancement into adulthood, it is important to understand the specifics of how EE influences adolescent behavior, as well as neural development, with the anticipation of future studies and the continuation of research.

Social Preference Task

Hypothesis 1 predicted that adolescent rats with a history of enrichment would show no preference to either stimulus rat during the SPT, and the non-enriched adolescent control rats would show preference to the novel stimulus rat. This hypothesis was partially supported. Enriched animals did not show significant preference toward either stimulus rat, as predicted. This finding suggests that EE was effective in providing rats with an environment that was complex and arousing enough to suppress their curiosity when later introduced to a new social stimulus, as demonstrated in the SPT. This idea is supported by research completed by Woods (1962) where it was discussed that a subject's immediate prior housing arrangement plays a large role in its subsequent perception of novelty. Similarly, this interpretation of the impact of EE on future behavior is also in line with Mora-Gallegos and Fornaguera (2019) who found that experience in a complex environment is directly related to an animal's spatial ability and habituation to novelty. The Mora-Gallegos study suggests that certain regions of the HPC play an important role in spatial tasks, providing animals with maps of their environments. Thus, the more maps an animal has stored, or the more complex each map is, the greater likelihood of them classifying a novel environment as familiar and demonstrating an unimpressive behavioral response (Mora-Gallegos & Fornaguera, 2019). Although not statistically significant, the enriched animals did, on average, spend 41% more time interacting with the familiar stimulus rat than with the novel rat, which can be explained by the emotional component of the SPT. During the first trial of the SPT, the enriched rat may have formed an emotional memory or connection with the stimulus rat, who would later be the familiar stimulus rat during the second trail of the SPT. The BLA has a crucial job in contributing emotion to memory (Sah et al., 2003; Vazdarjanova & McGaugh, 1999) and was found to be active in all animals and highly active in

enriched rats. Additionally, the finding that non-enriched animals did not show preference to the novel stimulus rat might be explained by their desire for broad social interaction (i.e., with both stimulus rats) as adolescents (Guyer et al., 2016; Kilford et al., 2016). As discussed, adolescence is a phase where among other behaviors, heightened social interaction between animals is observed (Spear, 2000). Because of this, these rats may have elected to engage themselves with both stimulus rats, providing for a more comprehensive social experience. Additionally, the introduction of a singular novel social stimulus may have not been exciting enough for the rat to solely commit themselves to this particular animal. Perhaps a more unique stimulus was needed in order for the animal to favor a risky decision: for example, significant engagement with the novel stimulus rat over a comprehensive social interaction, or spending more time with both stimulus rats.

Furthermore, the discovery that enriched males displayed the most interest in either stimulus rat was unanticipated. Enriched males spent more total time and more time per contact with either stimulus rat than did non-enriched males, enriched females, and non-enriched females. Although an interaction between EE and sex was not initially hypothesized, it can be predicted that its significance emerged from a variety of neural, hormonal, and developmental differences that occurs during adolescence. Sumiyoshi et al. (2017) used voxel-based morphometry (VBM), a technique that utilizes MRI to compare and measure brain tissue concentration, to better understand morphological changes in the rat brain. Their longitudinal study found that over time and in comparison to females, the male rat brain had greater inflations of total gray matter volume, as well as larger occipital cortexes, amygdalae, hippocampi and cerebella. Not only were these changes observed using VBM, but they were also consistent with and supported by previous neuroanatomy research. Cooke et al. (2007) and Johnson et al. (2008)

state that male amygdalae have a greater number of neurons, more complex astrocytes, greater dendritic branching, as well as more neurogenesis and astrogenesis. Additionally, Galea (2008) states that neurogenesis occurs uniquely in male and female hippocampi due to the inflection of gonadal hormones. These discrepancies in brain morphology provide reason behind the observed differences in behavior between sexes during the SPT. Sumiyoshi et al. (2017) also note that female rats undergo puberty at a much earlier age than male rats, generating further neuroanatomy inconsistencies between sexes.

Amygdala and Hippocampus

Hypothesis 2 stated that adolescent rats with a history of enrichment would have a significantly greater number of neurons in BLA and CA2 activated by performance of the SPT than adolescent control rats. This hypothesis was partially supported. There were increases of activated, c-FOS containing neurons observed in both the BLA and CA2 of enriched animals, however, that increase was only statistically significant in BLA (201%) and not in CA2 (16%). The inflation of active neurons observed in the BLA of enriched animals can be explained by findings of Okuda et al. (2009), which suggest that one week of EE can increase the overall number of progenitor cells found in the amygdala. Meaning, one week of EE can advance cell proliferation and differentiation in the amygdala by significant levels, as well as inhibit apoptosis. The extremely large difference in the number of active neurons present in the BLA of enriched animals in comparison to control suggests that EE may have promoted cell proliferation in BLA resulting in more neurons to be activated by the SPT experience.

The surprising minimal gain in the number of active neurons observed in the CA2 of enriched animals after social interaction was inconsistent with most literature. It was predicted that the SPT would promote greater enhancement of active neurons, as this is what previous

research has found (e.g., Alexander et al., 2016; Stevenson & Caldwell, 2014). Because CA2 plays such an essential role in an animal's social memory and in social recognition, it was thought that heightened exposure to such instances would generate a substantial amount of neural activity (Alexander et al., 2016). However, because CA2 is specific to social settings, it is possible that the 3 min trial of the SPT was not long enough to evoke enough neural activity to enhance firing in areas, like CA2, important for social recognition and memory. The enriched animals were accustomed to 90 min EE sessions, which included five other conspecifics, so a brief interaction among two stimulus rats in a simple environment may have failed to produce raw material for meaningful social memories. It is perhaps important to mention that even though statistically significant changes in neural activity were not observed in CA2, there was a subjective but obvious enhancement of activity observed in the piriform cortex – a region of the brain that is related to an animal's sense of smell. As discussed by Stevenson and Caldwell (2014), social memories are partially formed through an animal's olfactory system. The observation of activated (i.e. c-FOS containing) neurons in piriform cortex is indicative that cues needed for the formation of social memories were being processed during the SPT. Perhaps the information could not be consolidated, using CA2 in the process, during the 3 min trial. Although the number of neurons in the piriform cortex was not counted or measured, the density of neural activity was noticeable.

Limitations and Future Directions

The current study was conducted using a relatively small sample of subjects. Had a greater number of adolescent rats been involved, there would have been more power is statistical analyses and possibly more statistically significant results. Additionally, it is impossible to know whether housing rats in groups of three had any impact on their response to later social

interaction. It would have been interesting to include social isolation as an experimental group to determine whether those animals were more or less influenced by EE. Lastly, it may be worthwhile to observe risk-seeking and exploratory drive through an additional task such as the elevated plus-maze, as used by Marci et al. (2002). This would provide greater understanding of a subject's fear response and how it may differ from their behavioral response toward novel physical and social stimuli.

Subsequent analogous studies may consider other methods of marking neural activity, as c-FOS is limited in its ability to identify specific types of neurons. Further, examining neural cell types other than neurons would be interesting. Cooke et al. (2007) and Johnson et al. (2008) discussed that male amygdalae are composed of more complex astrocytes than female amygdalae, however, this would have been impossible to indicate in the current study while using such a broad neural activity marker and not staining specifically for glial cells. Continuing with sex differences, future research might look at running male and female rats in varied ages during adolescence, as females undergo puberty at an earlier time which causes subsequent difference in neuroanatomy, neural chemistry, and hormones (Sumiyoshi et al., 2017). Lastly, prospective studies should consider including juvenile and adult rats to observe baseline neural chemistry and behavior, as well as the influence that EE has on either component long-term.

Summary and Conclusions

This study provides insight into how enriching environments can persuade an animal's behavior and neurological structure. Specifically, the repetition of enriching experience, each with the opportunity for novel occurrences, as an adolescent seems to decrease risky social behavior at the end of that time as well as alter neural activity suggesting changes in synaptic strength. These findings provide information about adolescence in general and permit comments

about human beings. It may be that allowing adolescents to experience new situations and environments may actually be beneficial, as their probability to engage in risky or dangerous behaviors at a later date is decreased, owing at least in part to their neural networks being enhanced. These changes will hopefully extend into adulthood and exist as a long-term outcome of enrichment. For humans, social stimulation might include meeting different people or interacting with new peers, while physical stimulation may involve traveling to different cities or altering living arrangements. Although this study is impossible to replicate in human beings, it may be interesting to look for associations between a human's environment and their subsequent behavioral response to novel social interactions. When or if applicable, post-mortem studies might include staining for neural activity or generally looking at brain volume and cortical thickness, as these are known indicators of enhanced neurogenesis and cell proliferation in rats (Simpson & Kelly, 2011), and may therefore also apply to the human species. The headway that analogous studies make in this area of research should seek further analysis of behavior and neural function, or the inclusion of additional variables. Overall, continuation of this type of research is essential in promoting increased knowledge and recognition of the human adolescent.

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Table 1. Time (seconds) Spent with Novel vs. Familiar Stimulus Rat during Trial 2 of the SPT

Condition	Stimulus Rat	N	M	SD
EE	Familiar	12	12.17	5.20
EE	Novel	12	7.83	5.61
Control	Familiar	12	9.42	3.00
Control	Novel	12	8.67	4.74

Note: There was no significant difference in the time enriched rats spent interacting with either stimulus rat. Additionally, non-enriched rats did not show preference to the novel stimulus rat.

Table 2. Time (seconds) Spent with both Stimulus Rats during Trial 2 of the SPT

Sex	Condition	N	M	SD
F	EE	12	16.25	6.90
F	Control	12	13.25	6.36
M	EE	12	44.08*	22.20
M	Control	12	14.92	7.46

Note: There was a significant increase in the total time that enriched males spent with either stimulus rat; *$p < .05$.

Table 3. Time (seconds) Per Contact Spent with Both Stimulus Rats during Trial 2 of the SPT

Sex	Condition	N	M	SD
F	EE	12	2.03	0.91
F	Control	12	2.08	0.82
M	EE	12	4.94*	2.25
M	Control	12	1.25	0.48

Note: There was a significant increase in the time per contact that enriched males spent with either stimulus rat; *$p < .05$.

Table 4. Mean Neurons Expressing c-FOS Protein for Enriched and Non-Enriched Rats in BLA and CA2

	BLA			CA2		
Condition	N	M	SD	N	M	SD
EE	8	23.75*	12.41	8	9.00	2.92
Control	8	7.88	2.26	8	7.75	2.22

Note. There was a significant increase in the proportion of mean neural densities found in BLA of enriched animals; *$p < .05$. Abbreviations: BLA, basolateral amygdala; CA2, *Cornu Ammonis* 2 of the hippocampus.

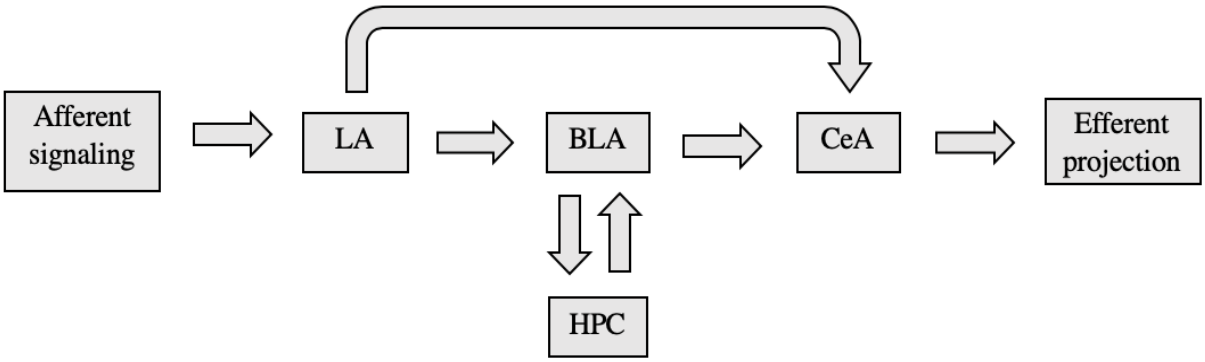

Figure 1. Circuitry of the rat amygdala with hippocampus. Of particular importance includes the bypass from LA to CeA, and the bridge between BLA and HPC. Abbreviations: LA, lateral amygdala; BLA, basolateral amygdala; CeA, central amygdala; HPC, hippocampus.

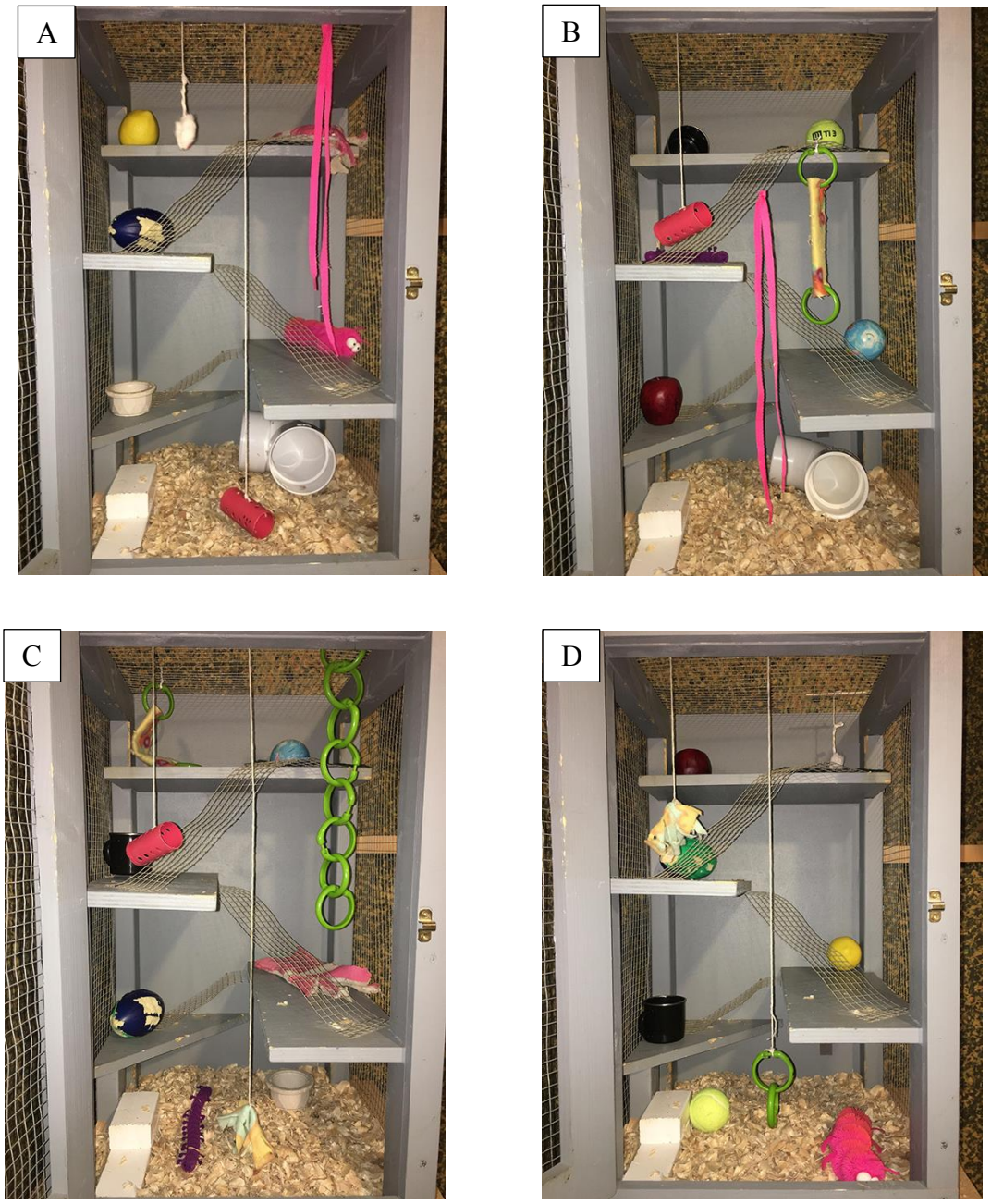

Figure 2. All four female enrichment cage set-ups (A-D). Each set-up includes various toys, ramps, swings, etc. All male enrichment cages mirrored the female cages. In order to maintain novelty, the cage set-ups were rotated after each enrichment session.

Figure 3. The 63 X 42 X 45 cm (w X d X h) wooden box with aspen bedding on the floor for the social preference task (SPT).

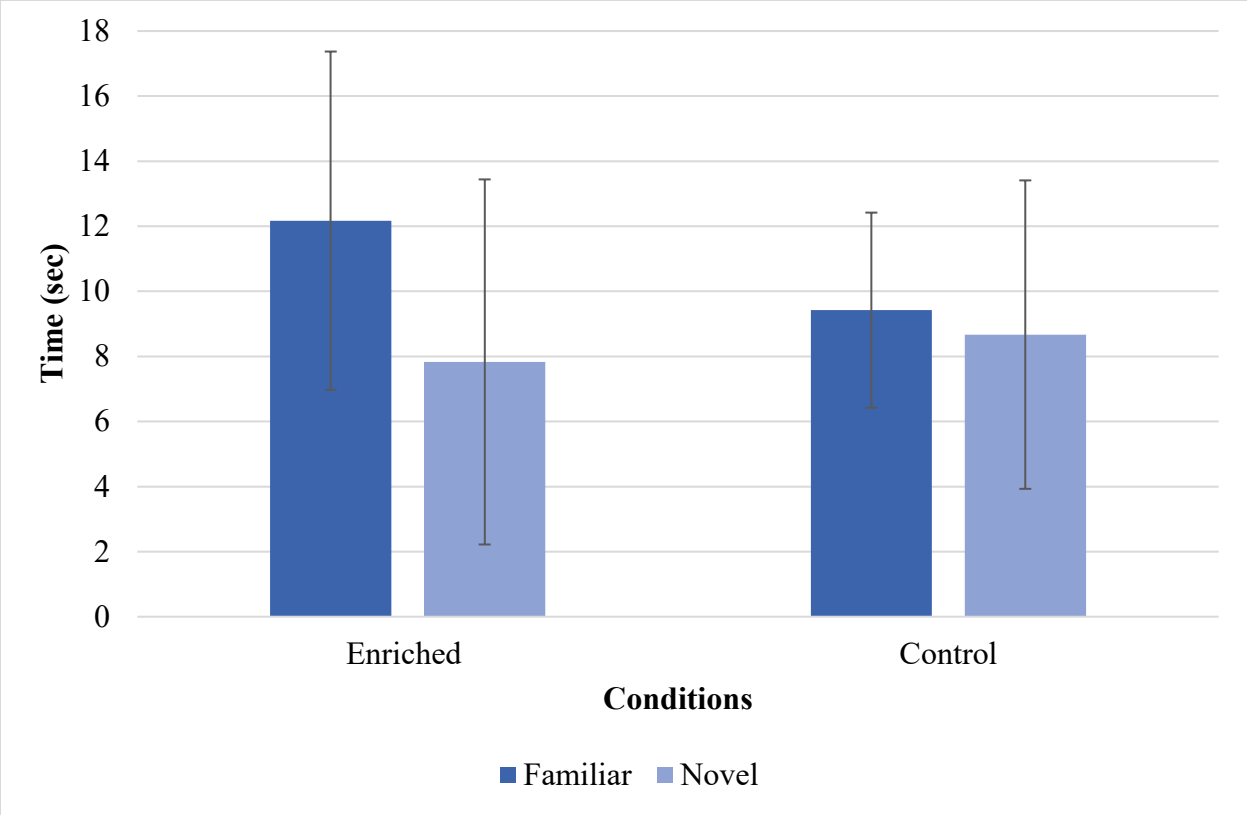

Figure 4. Mean time spent with the familiar vs. novel stimulus rat across conditions on Trial 2 of the social preference task. Error bars represent standard deviations (SD). There was no significant difference in the time enriched rats spent interacting with either stimulus rat. Additionally, non-enriched rats did not show preference for the novel stimulus rat.

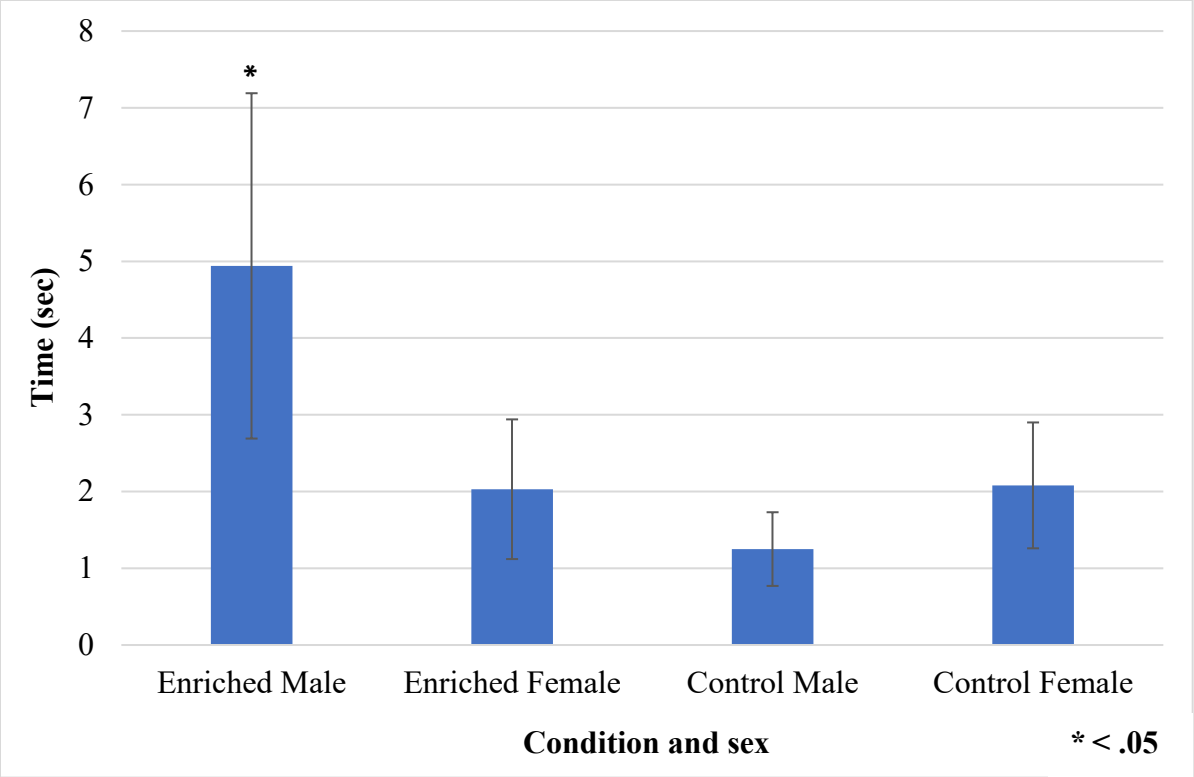

Figure 5. Mean total time spent interacting with either stimulus rat across conditions and sexes during Trial 2 of the social preference task. Error bars represent standard deviations (SD). There was a significant increase in the total time that enriched males spent with both stimulus rats, which is indicated by *.

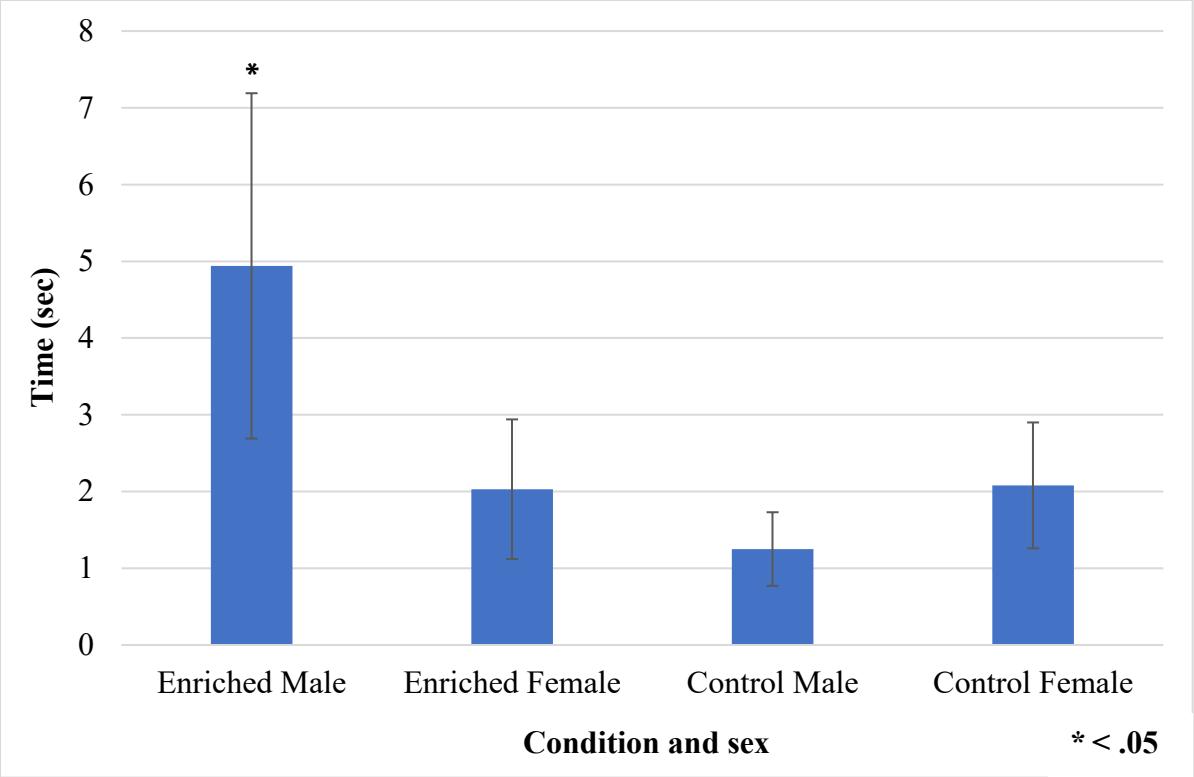

Figure 6. Mean time per contact with either stimulus rat across conditions and sexes. Error bars represent standard deviations (SD). There was a significant increase in the time per contact that enriched male rats spent with the stimulus rats, which is indicated by *.

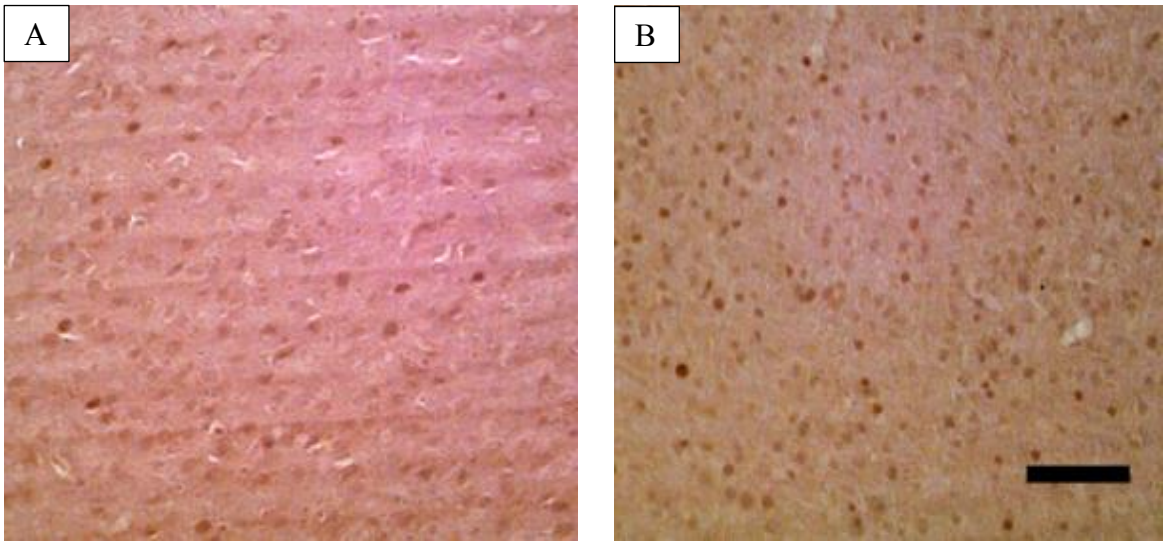

Figure 7. (A) BLA of control subjects. (B) BLA of EE subjects. EE subjects showed a 201% increase in the number of active neurons. Abbreviations: BLA, basolateral amygdala.

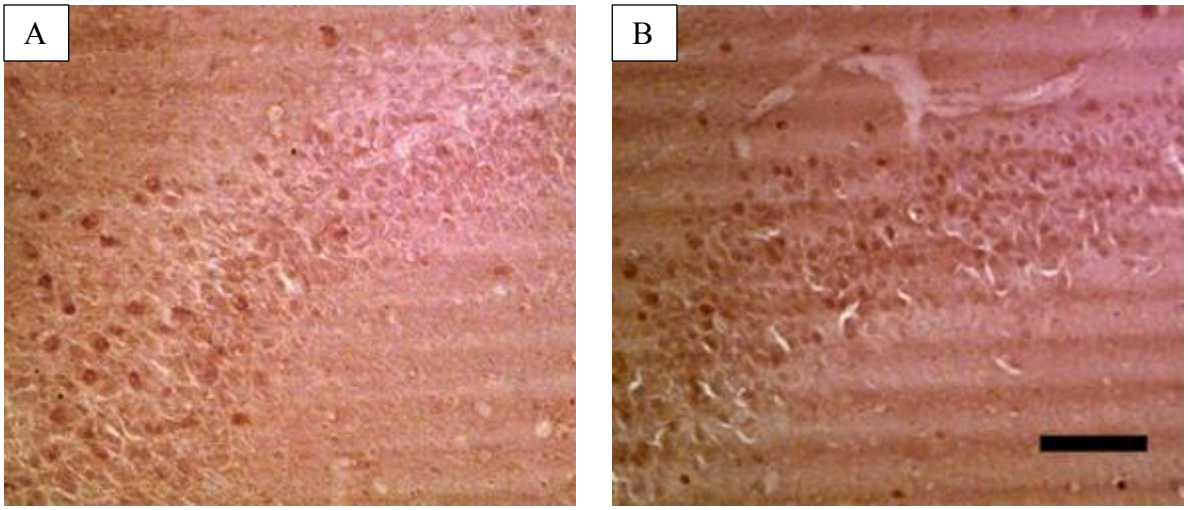

Figure 8. (A) CA2 of control subjects. (B) CA2 of EE subjects. EE subjects showed a 16% increase in the number of active neurons. Abbreviations: CA2, *Cornu Ammonis* 2 region of hippocampus.

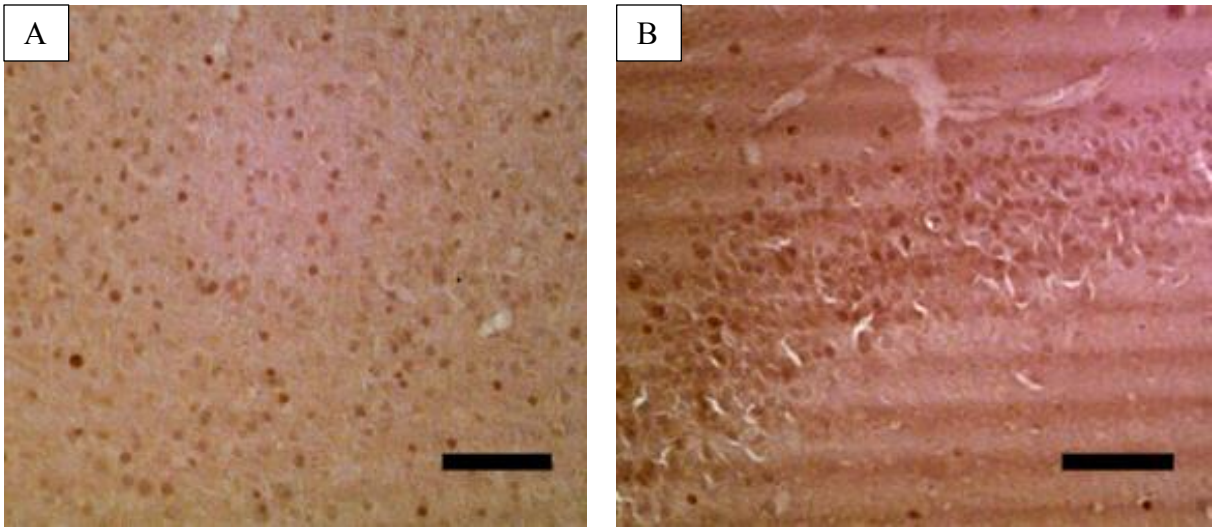

Figure 9. (A) BLA of EE subjects. (B) CA2 of EE subjects. BLA showed 164% more active neurons in BLA than in CA2. Abbreviations: BLA, basolateral amygdala; CA2, *Cornu Ammonis* 2 region of hippocampus.

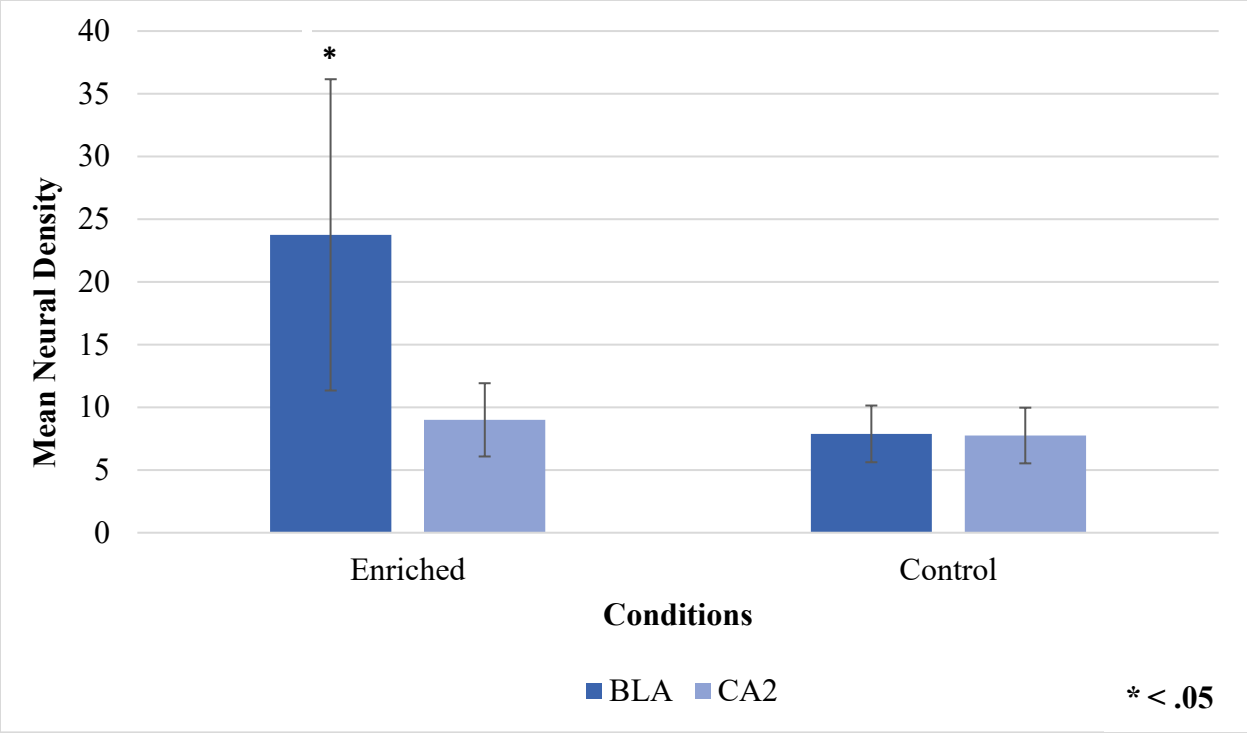

Figure 10. Mean neural densities of c-FOS positive neurons in BLA and CA2 of each condition. Error bars represent standard deviations (SD). There was a significant increase in the mean density of active neurons found in BLA of enriched animals, indicated by *. Abbreviations: BLA, basolateral amygdala; CA2, *Cornu Ammonis* 2 region of hippocampus.